FINAL REPORT

This document is a final report submitted to the NIH Neural Prosthesis program, summarizing results obtained in Contract NS 5-2322, "Surface Modification for Biocompatibility", awarded to co-Principal Investigators David C. Martin in Materials Science and Engineering, College of Engineering, and K. Sue O'Shea, Anatomy and Cell Biology, Medical School, at the University of Michigan. The objectives of this work were to: a) Select candidate surfaces to enhance the mechanical stabilization of silicon devices implanted in the central nervous system, b) Select candidate surfaces to promote neurite outgrowth to specific regions of implant microstructures, c) Develop coating technology, physical and chemical characterization of surfaces, and evaluate coating stability, d) Develop a cell culture model of neurons and glial cells derived from the mammalian cortex for initial *in vitro* evaluation, and e) Pursue *in vivo* studies and collaborations with other workers. In this report we discuss what was achieved, what was not achieved, and what we believe would be useful to pursue in future research and development efforts. We also include a list of publications supported by this work, as well as copies of these publications.

By specific aim of the original Statement of Work:

a). Select candidate surfaces that are likely to enhance the microscopic mechanical stabilization of a microstrucure implanted within the central nervous system.

We specifically proposed to manipulate the phase stability of protein polymers during processing. Their three dimensional microstructure will be tailored to support a cohesive cellular device / tissue interface. Aims included drug delivering porous coatings, which promote specific neural ingrowth and limit gliosis, and rough coatings which increase surface area and provide niches for cellular attachment.

What we did:

The materials which were the focus of this work were genetically engineered protein polymers, commercially available from Protein Polymer Technologies, Inc., which combine the structural peptide sequences from silk (GAGAGS) and elastin (VPGVG) with bioactive binding sites from extracellular matrix binding proteins such as fibronectin (RGD) and laminin (IKVAV).

We evaluated a number of schemes for preparing microstructured thin films with elements to promote the formation of roughness on the nanometer length scale which would maximize surface area and provide for regions to promote the ingrowth of cells. We focused considerable attention on an electostatically-mediated fiber deposition technique (electospinning), which proved to provide a reproducible means for creating a porous film in which the microstructural elements of the polymer could be precisely controlled. We found that films composed of beads, hairy beads, or uniform filaments could be created by changing the concentration of the polymer in solution. The lateral profile of the fibers and density of the film could be tailored by changing the separation

distance between the syringe and substrate. Typical conditions used for film deposition were a potential of 7 kV, separation distance of 20 mm, solution concentration of 150 mg/ml polymer in formic acid solvent, and deposition time of 5 seconds. This results in a fibrous film with filament diameters on the order of 200 nm and a thickness of approximately one micron.

Finite element models of the probes implanted in brain were obtained, and used to evaluate the anticipated modes of deformation for an implanted probe. Comparisons were made between rigid and soft substrates, and between probes with poor adhesion or strong adhesion to the tissue. The relative motion of the brain was simulated in both shear and "poking" deformations. It was found that severe strains could develop near the surface of the pia in shear, and near the tip of the probe in poking. Also, delamination of the probe from the tissue was predicted in the case of poor adhesion. These problems were found to be reduced with stronger adhesion between the probe and the tissue and by reducing the stiffness of the probe material.

Considerable effort was invested to evaluate the relationship between deposition conditions, film morphology, biological activity, and electrical transport. The porous, filamentous films which were created provide for the adhesion of cells from the tissue; mediate the large differences in mechanical properties between the rigid silicon substrate and the soft neural tissue; and maintain the ability to transport electrical signals. We also established that a number of biological active agents could be incorporated into the films including several antibiotics (amoxicillin, bacitracin, cephalosporin C, erythromycin, hygromycin B, kanamycin, neomycin sulfate, nystatin, tetracycline), caffeine, aspirin, and nerve growth factor (NGF).

A concern voiced at one of the annual meetings by a neurosurgeon was that he observed as many as 5% of his patients develop localized infections following microelectrode implantation. In response to this comment, we incorporated an antibiotic (kanamycin) into the polymer, coated coverslips with 25% antibiotic:75% SLPF and placed them in 12 well plates containing growth medium which we innoculated with bacteria. Bacteria were killed after as little as 6 hrs *in vitro* in antibiotic containing wells, but not in wells containing SLPF alone (QPR, 10/31/96).

One of our primary interests in using protein polymers was the ability to deliver growth factors that might be involved in neuronal survival near the implant. Toward this end we needed to determine if the processing of the polymer would destroy any activity of a bound growth factor. In attempting to find an assay which would cause an identifiable response of a neuron to a growth factor, we chose to examine the ability of SLPF to which nerve growth factor was added to cause the differentiation of PC12 cells. PC12 are a rat pheochromocytoma (tumor) cell line which grow with an undifferentiated morphology *in vitro*. When exposed to growth factors such as nerve growth factor *in vitro*, they differentiate, extending a neuronal process and cease dividing. We rapidly mixed 100 micrograms of NGF in 100 microliters of SLPL, then spun the blend onto glass coverlips which were then washed, and briefly UV light surface sterilized. In order to determine if the NGF/SLPL blend was biologically active, PC12 cells were plated onto

NGF/SLPL coated coverslips. Controls were grown on SLPL alone or on SLPL to which 50 ng/ml NGF was added (to the culture medium). After 4-5 days the blend supported robust neurite outgrowth similar to that seen when NGF was added directly to the cultures. SLPF alone did not promote neuronal differentiation, and we did not observe any evidence of toxicity in any of these cultures. We believe that this series of experiments demonstrates that growth factors and antibiotics can be delivered in an active form into a highly localized region via the protein polymer blend (QPR 11/17/95).

What we didn't do:

Bioactivity of rough coatings:

We established that variations in surface roughness induced by processing could be characterized by atomic force microscopy, and that this roughness was length scale dependent. For beaded coatings, thin films were found to be smooth at large lengths and rough at small length scales, where thick coatings were smoother at small length scales, yet rougher at large length scales. The implications of these subtle changes in structure on biological activity remains to be established, although with a means now established for characterizing these details in microstructure it should be feasible in future efforts.

Anti-gliosis coatings:

One of the concerns with indwelling devices in the CNS is the reaction of glial cells to the implant itself; glial cells often divide and attempt to "wall off" the implant from the CNS, thereby producing additional damage. We intended to deliver coatings which might impede the formation of this "scar" such as an anti-mitotic drugs via the coating. Since such scarring was not observed in our implants, this was not attempted.

b). Select candidate organic surfaces that are likely to enhance the close approximation of neurons or neuronal processes to specific regions of implanted silicon microstructures.

Specifically, we proposed to seed neonatal Schwann cells onto regions of the implant to provide growth factors and a substrate which promotes neurite outgrowth. A similar approach may be pursued acellularly by generating a porous coating based on extracellular matrix proteins selective for neurons, which can deliver growth factors to specific areas of the implant.

What we did:

Coated polypropylene suture with SLPF, SLPL, SELP, SLPL-Schwann cells, implanted them into Guinea pig cortex and evaluated the cellular response to them after 3 weeks, 12 weeks, and 9 months. The goal of this series of experiments was to use polypropylene suture to deliver the coated materials into brain and determine first if there was any deleterious effect of the coating, and second if there was any increase in the number of neurons or neuronal processes toward the implant. Initially, these studies were

carried out entirely by the Kresge group, who found that polypropylene when embedded in methacrylate could not be sectioned using glass knives, as there was not a match in hardness between the softer methacrylate and stiffer suture. We carried out a series of experiments to determine what resin was a proper match, and the rest of the studies were carried out using Spurr's resin which allowed the suture to be retained in the section, thereby providing the ability to observe the direct interaction of cells with the coating material. Results from these studies indicated that there was surprisingly little reaction by the neuropil to these coatings (QPR 10/31/95, 10/31/96) at any stage examined. There was no real indication either that there was improvement in neuronal position or behavior relative to the implant. At this time, we decided that the coatings were not toxic, and to better evaluate cell response to them, we needed to employ a technique that would let us carry out immunohistochemical localization of cell-type restricted proteins to identify them in thick (ground) sections.

Since that time, in collaboration with Chris Edwards in the Cell Biology Laboratory of the Department of Anatomy and Cell Biology, coated probes were placed in Guinea pig cortex, embedded in resin and ground sections taken. Prior to embedding blocks of cortex containing probes can be incubated with DNA labels (to identify cells near the probe), and we anticipate that it will be possible to also carry out en block immunohistochemical localizations prior to embedment and sectioning to obtain more information about the cellular interface with the probe (and coating). All coatings: uncoated, SLPL, SLPF have been implanted, and embedded. We are awaiting "sectioning" of the probes to produce a detailed map of cell-probe surface interactions by laser confocal microscopy (QPR 10/31/97, 1/31/98, and ongoing). This technique has the considerable advantage of allowing the entire electrode shaft to be imaged, and we have already observed some alterations along one side of the implants suggestive of stress.

What we didn't do:

We have yet to demonstrate that growth factors can be delivered to cells via SLPL *in vivo*. However, the success of our *in vitro* results indicates that this is completely reasonable.

c). Develop or adapt available methods to bond the selected organic molecules to a silicon dioxide surface like the surface of a micomachined electrode and to chemically characterize these surfaces before and after protein adsorption.

Specifically, we proposed to characterize the microstructure using AFM, TEM, ESEM, adhesion to various substrates using scratch testing. Alter surface patterning using microlithography, photolithography, or masking techniques.

What we did:

Information about protein polymer film morphology was obtained by optical microscopy, scanning electron microscopy, transmission electron microscopy, and wide

angle X-ray scattering. Quantitative information about the sample surface morphology was obtained by atomic force microscopy, and it was observed that the roughness of the film was a function of spatial frequency (from 0.02 to 2 μm^{-1}). Nanoindentation was used to evaluate the near-surface mechanical properties of the films as a function of their microstructure. Mechanical properties were also evaluated by scratching and by deformation of porous polymer films adhered to deformable copper substrates. Impedance spectroscopy of the films was obtained as a function of temporal frequency (from 10 Hz to 1 MHz).

Patterned films of protein polymers were created by depositing the films onto silicon substrates with patterned photoresist. It was found that removal of the photoresist left the protein polymers behind on the substrate, and this could be done with both beaded or filamentous morphologies.

Photolithographic patterning was also carried out by a post-doctoral research associate, Dr. Libby Louie. After several attempts to use stamping technology, Libby traveled to the Cornell Nanofabrication Laboratory to create a mask with very defined properties to allow us to reliably produce pattened protein polymer stripes on glass coverslips using microlithographic techniques. (QPR 7/31/97, 10/31/97). Libby was able to create very accurate patterns of protein polymer stripes (2, 10, 50 micrometers in width) with spaces of 2, 10, and 50 micrometers) between the stripes. The goal of this series of studies was to present neurons and glial cells with a forced choice of substrate: either non-coated glass, SLPL, SLPF, or SELP (or their native proteins, laminin, fibronectin, or elastin). We could examine cell morphology, and preference by various cells of one type of protein versus another, with the long term goal of directing cells to the active zone of the probe surface, and causing them to actively avoid (SELP coating) other regions where cell adhesion is detrimental (e.g., pial surface).

In addition, each series of stripes incorporated a central zone where cells to be tested were held within a "corral" to allow them to equilibrate to culture conditions, force a choice in substrate, and to give us a starting point to determine parameters such as distance traveled (from the start point). These studies have provided a real wealth of data, and some cell behavior parameters are still being analyzed using NIH image. In addition a NSF summer student in the lab, Ms. Carla Kovacs, examined the nature of the cell-substrate interaction using antibodies to focal adhesion kinase, and to cytoskeletal proteins present in the region. These studies demonstrated that protein polymers are not toxic to neurons or to glial cells in vitro; that glial cells require an attachment zone of at least 10 micrometers, that neuronal cells are far more differentiated on SLPL substrates than on SLPF; that glial cells can attach to SLPL, but spread much better on SLPF; SELP is not adhesive for either cell type.

What we didn't do:

We have yet to pattern protein polymers directly onto neural probes using microlithography. However, we have shown that simple masking can be used to direct the polymer to regions of specific interest. Also, we have found that the electric field can

be used to direct the deposition of the polymer coating to more conducting regions of patterned silicon substrates.

d). Develop a cell culture or other suitable model of mammalian cortex and investigate the growth and adhesion of neurons, glia, microglia, and other cells present in the nervous system on substrates coated with the selected surfaces.

Specifically, we proposed to culture neurons and glial cells from developing cortex. Culture cells on polymers and examine behaviors, culture cells on polymer-glial cell-cell substrates. Transfect cells to express growth factors or cytokines required for their survival.

What we did:

We cultured neurons (neuro 2A cells) and glial (Schwann cells) cells on polymers and examined their morphology and behavior as described in detail in c) above. We also developed methodologies to transfect pluripotent embryonic stem cells with neurodetermination genes for transplantation. We decided to develop a system that we would provide better access to cells, and could be used to express neurotransmitter, growth factor genes, as this is currently difficult/impossible in primary cells. Although most labs use neural stem cells derived from the ventricular zone of the cortex for this purpose, we differentiated embryonic stem cells down a neuronal lineage for these transplantation studies. Embryonic stem (ES) cells are derived from the blastocyst inner cell mass, and remain totipotent when cultured in the presence of leukemia inhibitory factor, or on the surface of fetal fibroblasts. Remarkably, these cells retain the ability (even after genetic manipulation *in vitro*) to continue their development when re-introduced into the blastocyst, and have been used extensively to create mutations in genes via gene targeting and homologous recombination (gene knock out). Because they are totipotent and proliferate extensively *in vitro,* they are ideal for studies of cell/gene delivery. We have carried out a number of investigations to optimize the growth factor/substrate characteristics required for neuronal differentiation of these cells, and have developed a "cocktail" that reliably results in neuronal differentiation after periods of 48-72 h *in vitro*. In addition Dr. Theresa Gratsch has recently cloned the mouse neural induction gene, noggin. When noggin is expressed in ES cells, they very rapidly down-regulate expression of stem cell genes and up-regulate expression of neural differentiation genes, including genes of the bHLH family of transcription factors, the neuroDs. We have coexpressed marker genes such as the jellyfish green fluorescent protein, so these cells can be identified, and have begun preliminary studies in which noggin/GFP neurons have been injected into mouse embryos growing in whole embryo culture, as well as into the early cortex of mouse embryos (d11) via exo utero surgery. We believe this approach is significantly better than using primary cultures of neurons and glial cells because the cells are derived prior to implantation, so they do not express antigens of the MHC family, and they can be transfected to express growth factors, signalling molecules, differentiation inducing factors, and proteases prior to transplantation into the region of interest. The noggin neurons, and also neuroD2 and

neuroD3 expressing neurons we have developed in the lab are very primitive and should respond to the brain by developing further; hopefully in a region specific manner. With the recent development of human embryonic stem cells, similar experiments might soon be possible in cases of human degenerative conditions which could be improved by implantation.

What we didn't do:.

We didn't use primary cultures of neurons and of glial cells, because we believe that the primitive neurons formed by differentiation of ES (others have developed protocols for glial cell differentiation) are superior for this purpose. This is because they are primitive, are an unlimited source of cells, and can be transfected to express growth factors required for survival in the region of the implant.

e). Cooperate with other investigators in the Neural Prosthesis Program by coating microelectrodes (estimated 50 over the contract period) with the most promising materials for in vivo evaluation as directed by the NINDS Project Officer.

What we did:

Stainless steel electrodes for implantation into Rhesus monkeys were coated from D. Kipke and A. Schwartz at Arizona State University for *in-vivo* investigations. The study was not successful at achieving recordings, although the problems did not seem to be associated with the coatings.

Both arrowhead and sieve probes were coated for A. Mensinger and S. Highstein at Washington University, who found success in their work with the toadfish *Opsanus Tau*. Single neural activity was recorded with high fidelity from seven unrestrained fish within 30 to 60 days following implantation. Increased numbers of neurons were observed growing through the holes of the sieve as compared to the uncoated controls.

RECOMMENDATIONS FOR FUTURE RESEARCH AND DEVELOPMENT:

1. Continue to develop better microscope visualization technologies such as the EXAKT technique to allow rapid, analysis of the probe–cell interface.
2. Synthesize additional protein polymers; containing anti-mitotic agents, possibly anti-amyloid constructs; with different cell specificities, and with the capability to facilitate ionic transport.
3. Deliver primitive neurons transfected with region specific growth factors with the probe.
4. Examine the biological response of coatings with systematic variations in roughness at different length scales.
5. Examine the mechanical properties of coatings with different variations in filament morphology and controlled gradients in structure.
6. Consider additional materials at the probe-tissue interface such as electronically conducting polymers (polyaniline, polypyrrole) near the probe, and ionically conducting polymers (polyethylene oxide, porin) near the tissue. Combine these as blends with the bioactive protein polymers to promote favorable biological interactions.
7. Confirm that any new proposed coating schemes remain electronically viable by Impedance Spectroscopy.

Publications supported by NIH Contract NS 5-2322:

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L. K. Louie, D. C. Martin, K. S. O'Shea, and M. Hortsch, "Glial and Neuronal Cell Response to Substrates Patterned with Multiple Native and Synthetic ECM Proteins", Society for Neuroscience Abstracts, (1997).

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James E. Raynolds, Michael A. Johnson, and David C. Martin, "Fiber Formation from Fluid Cones Induced by Electric Fields", in preparation, intended for submission to the Journal of Applied Physics, (1999). Draft of abstract included.

Michael A. Johnson and David C. Martin, "X-ray Diffraction Studies of Bombyx mori Silk Fiber and Silk-Like Protein Polymers", in preparation, intended for submission to Materials Science and Engineering, (1999). Draft of abstract included.